Kidney International, Vol. 59 (2001), pp. 147–159

Antineutrophil cytoplasmic antibodies to proteinase 3 in Wegener's granulomatosis: Epitope analysis using synthetic peptides

YMKE M. VAN DER GELD, ARJAN SIMPELAAR, RUURD VAN DER ZEE, JAN WILLEM COHEN TERVAERT, COEN A. STEGEMAN, PIETER C. LIMBURG, and CEES G.M. KALLENBERG

Department of Internal Medicine, University Hospital Groningen, Groningen, and Institute of Infectious Diseases and Immunology, University of Utrecht, Utrecht, The Netherlands

Antineutrophil cytoplasmic antibodies to proteinase 3 in Wegener's granulomatosis: Epitope analysis using synthetic peptides.

Background. Antineutrophil cytoplasmic antibodies (ANCA) to proteinase 3 (PR3) are strongly associated with Wegener's granulomatosis (WG) and are thought to be involved in its pathogenesis. Levels of PR3-ANCA do not always correspond to clinical disease activity nor to functional effects of these antibodies in vitro, suggesting differences in epitope specificity. To define relevant epitopes for PR3-ANCA, sera of WG patients were analyzed on their reactivity to linear peptides of PR3.

Methods. Fifty linear peptides of 15 amino acids in length with an overlap of 10 aa spanning the entire PR3 sequence were synthesized. Sera of 27 WG patients with active disease and 27 age- and sex-matched healthy controls, eight anti-PR3 monoclonal antibodies (mAbs), and a rabbit anti-PR3 serum were tested by enzyme-linked immunosorbent assay for reactivity to PR3 peptides.

Results. Rabbit anti-PR3 serum recognized three distinct peptide areas, whereas none of the anti-PR3 mAbs bound PR3 peptides. Sera of both WG patients and healthy controls recognized a restricted number of PR3 peptides. Four of these peptide areas were recognized significantly more strongly by WG sera than by control sera. Sera drawn at the initial presentation of WG mainly recognized these peptides. Two of the recognized peptide areas were located near the active center of PR3.

Conclusion. A restricted number of epitope areas of PR3 are recognized both by WG patient sera and control sera. Four peptide areas were bound stronger by sera of WG patients at initial presentation than by healthy controls.

Wegener's granulomatosis (WG) is characterized by granulomatous inflammation in the upper and lower respiratory tract, systemic vasculitis affecting small blood vessels, and pauci-immune–necrotizing crescentic glomerulonephritis [1] together with the presence of antineutrophil cytoplasmic antibodies (ANCAs) in almost all of the patients [2]. The latter finding suggests an autoimmune pathogenesis. The target antigen for ANCA in WG has been identified as proteinase 3 (PR3) [3–5], which is one of the four serine proteases in the azurophilic granules of neutrophilic granulocytes and monocytes [6]. PR3-ANCA has been established as a specific marker for WG, and detection of these autoantibodies is helpful in the diagnosis and follow-up of WG patients [7, 8].

Although the etiology of WG is still unknown, several observations suggest that PR3-ANCAs are involved in the pathogenesis of WG [9]. First, titers of PR3-ANCA rise prior to a relapse of WG in many patients [7, 10, 11], but these data have been disputed by others [12, 13]. In addition, PR3-ANCA can bind to PR3 expressed on the cell surface of primed neutrophils and subsequently activate these cells. Upon activation, neutrophils produce reactive oxygen radicals and degranulate lysosomal enzymes, including PR3 [14, 15]. Finally, PR3-ANCA can interfere with PR3 proteolytic activity as well as with the binding of the natural inhibitor α1-antitrypsin to PR3 [16–18]. Data from a relatively small group of patients show that the previously-mentioned functional characteristics of PR3-ANCA correlate stronger with disease activity than the ANCA titer alone [18, 19]. These data suggest that differences in pathogenic potential of ANCA may be related to differences in epitope specificity of these antibodies.

Elucidation of the epitopes recognized by PR3-ANCA using recombinant or modified PR3 has proved to be difficult [20]. Antibody binding is abrogated by denaturing the antigen and by reducing its disulfide bonds [20, 21]. Despite this apparent requirement for an intact tertiary structure, there are three reports of PR3-ANCA

Key words: ANCA, inflammation, necrotizing crescentic glomerulonephritis, serine proteinase, linear epitopes on PR3.

Received for publication January 24, 2000
and in revised form June 13, 2000
Accepted for publication July 18, 2000

binding to linear peptides (abstract; Griffith, *Vasc Diffuse Lung Dis* 13:256, 1996) [21, 22]. Two reports identified distinct epitope regions that were bound by PR3-ANCA (abstract; Griffith, *Vasc Diffuse Lung Dis* 13:256, 1996) [22]. However, in one report, no binding of PR3-ANCA to linear peptides could be detected because of a high aspecific binding [21].

The former studies used peptides that were formed by coupling amino acids on pins [21, 22]; sequence and purity of these peptides can be checked only after removal from the pins. Using this system, sera of healthy and disease controls also bound to linear peptides of PR3 [21, 22]. Few clinical data were given on the sera tested, so any relationship between peptide recognition and clinical or laboratory features of disease could not be analyzed.

The aim of the present work was to identify possible linear epitopes on PR3 recognized by PR3-ANCA of defined patients with WG as well as of age- and sex-matched healthy controls. Furthermore, monoclonal antibodies (mAbs) to PR3 and a rabbit anti-PR3 serum were tested in order to compare findings in patients with those originating from an immune response to an exogenous antigen in experimental animals. Binding was determined by enzyme-linked immunosorbent assay (ELISA) in which soluble peptides were used, and sequence and quality control of peptides prior to use were performed. We provide evidence that PR3-ANCA sera as well as healthy control sera bind specifically to a restricted number of linear PR3 epitopes, two of which are located near the active site. The rabbit anti-PR3 serum recognized three distinct peptide areas that partly overlapped with the areas recognized by PR3-ANCA sera.

METHODS

Patients and controls

Sera were obtained from 27 patients with diagnosis of WG established according to clinical and histologic criteria [1]. Patients fulfilled the American College of Rheumatology criteria for WG [23]. All patients had active disease, either newly developed or relapsing at the time of testing. Active disease was defined as previously described [24, 25]. A distinction was made between limited ($N = 9$) and generalized ($N = 18$) disease activity based on the absence or presence of renal involvement, respectively. Most of the patients did not receive any immunosuppressive treatment at the time of serum sampling. One patient received pulse treatment of methylprednisolone. Sera from all patients were tested for ANCA by indirect immunofluorescence (IIF) on ethanol-fixed granulocytes and by ELISA for PR3, myeloperoxidase (MPO), and human leukocyte elastase (HLE; discussed later in this article). One patient (patient 1) had antibodies directed against PR3 as well as MPO. Serum IgG concentrations were determined by nephelometry. Clinical and laboratory data of the patients are outlined in Table 1. Sera from 27 age- and sex-matched healthy blood donors were used as controls. These sera were negative for ANCA by IIF, capture ELISA for PR3, MPO and HLE, and PR3-specific direct ELISA. Sera from three patients with early rheumatoid arthritis and sera from three patients with a relapse of systemic lupus erythematosus were tested as disease controls.

ANCA detection

Antineutrophil cytoplasmic antibodies were detected by IIF on ethanol-fixed granulocytes according to a standard protocol [2, 26] with minor modifications [27]. Patient and control sera were tested at serial twofold dilutions starting at a dilution of 1:20. Slides were read by two independent observers, and titers of $\geq$1:40 were considered positive for ANCA.

Specificity for PR3, MPO, or HLE was defined by antigen-specific capture ELISA, as previously described [28]. Sera were considered positive when values exceeded the mean + 2 SD of normal controls ($N = 50$).

Titers of PR3-ANCA were also assessed by antigen-specific direct ELISA [29]. In this ELISA, a standard PR3-ANCA serum was defined as 100 units, and the sera tested were calculated accordingly. Titers of six units or more were considered positive for PR3-ANCA, based on results of normal controls.

Purification of PR3

Proteinase 3 was purified according to the isolation procedure of Leid et al [30] with minor modifications. Enriched leukocyte populations were obtained from the Blood Bank of Groningen (The Netherlands) and were pooled and suspended in phosphate-buffered saline (PBS). Red blood cells were lyzed, and leukocytes were collected. Cells were disrupted by nitrogen cavitation at 4°C for 20 minutes at 350 psi (Parr Instruments Co., Moline, IL, USA). Next, granules were separated by discontinuous Percoll gradient (Pharmacia, Uppsula, Sweden), and α granules were pooled and disrupted in PBS with 2% Triton for 30 minutes at 37°C. The granule extract was applied to a Biorex-70 column (Bio-Rad Laboratories, Richmond, CA, USA). PR3-positive and HLE- and MPO-negative fractions were pooled and concentrated by aquazide II (Calbiochem, La Jolla, CA, USA). The concentrate was applied to a Superdex-75 column (Pharmacia). PR3-positive fractions were pooled and concentrated by aquazide II. PR3 was not contaminated with HLE, MPO, or cathepsin G as tested by ELISA and immunoblot (data not shown).

Antibodies

In this study, eight mAbs to PR3 were used. MAb 12.8 has been produced by Goldschmeding et al and is

Table 1. Patient characteristics

Patient no.	Sex	Age	Disease extension	Disease duration *months*	c-ANCA IIF titer[a]	PR3-ANCA *units*[d]	CRP *mg/mL*	Serum Creatinine *μmol/L*	Serum IgG *mg/mL*	Treatment
1	m	64	G	NP	>640	482	384	211	22.2	No
2	f	53	L	NP	160	227	37	81	22.5	No
3	m	78	G	NP	>640	13	163	136	13.2	No
4	m	72	G	NP	160	499	19	272	10.9	No
5	m	45	L	NP	80	312	96	97	14.9	No
6	f	59	G	NP	160	162	29	184	20.9	Pred, 20 mg[a]
7	f	70	G	NP	>640	361	122	480	13.3	No
8	m	58	G	NP	320	633	31	268	20.1	No
9	f	50	L	NP	160	16	111	74	15.2	No
10	f	24	G	NP	160	227	315	68	10.6	No
11	m	71	G	NP	320	247	190	243	23.1	No
12	f	71	L	NP	20	33	25	68	18.2	No
13	m	19	L	NP	160	353	4	76	13.2	No
14	m	47	G	NP	640	2933	39	113	16.2	No
15	f	56	G	NP	320	327	58	204	7.7	No
16	m	38	G	NP	640	324	38	106	8.9	Pred, 1000 mg[b] + CP, 175 mg[c]
17	m	80	G	7	320	272	23	455	11.7	Pred, 10 mg
18	f	52	L	13	40	37	8	88	11.0	Pred, 60 mg
19	m	67	G	15	160	12	100	143	19.6	Pred, 7.5 mg
20	m	54	G	20	320	900	<4	139	13.7	CP, 50 mg
21	m	71	G	21	>640	160	151	279	7.17	Aza, 25 mg
22	f	50	L	27	>640	258	21	73	10.6	No
23	m	50	L	82	160	25	7	102	12.4	No
24	f	71	G	118	>640	445	50	164	14.4	No
25	f	56	L	139	160	68	24	101	7.58	Aza, 150 mg
26	m	44	G	146	640	500	53	110	14.4	No
27	m	70	G	166	320	47	54	222	13.1	Aza, 100 mg

Abbreviations are: m, male; f, female; L, limited; G, generalized; NP, newly diagnosed patient; c-ANCA IIF titer, cytoplasmic ANCA titer measured by indirect immunofluorescence; PR3-ANCA, proteinase 3-antineutrophil cytoplasmic antibodies titer measured by direct ELISA in displayed units; Treatment, immunosuppressive treatment; No, no treatment; Aza, azathioprine; Pred, prednisolone/day; CP, cyclophosphamide.

[a] Treatment for 3 months

[b] Single pulse treatment 2 days before sampling

[c] Treatment for 2 days

[d] IIF titers of ≥1:40 were and PR3-ANCA titers of ≥6 units are considered positive for PR3-ANCA

available at the CLB (Amsterdam, the Netherlands) [3]. Dr. J. Wieslander (Wieslab, Lund, Sweden) provided MAbs 4A3 and 4A5 [31]. The mAbs Hz1F12 (abstract; Haung, *Clin Exp Immunol* 101:501, 1995) and MC-PR3-2 [32] were obtained from the late Dr. C.M. Lockwood (Cambridge, UK) and Dr. U. Specks (Rochester, MN, USA), respectively. MAbs PR3G-2, PR3G-3, and PR3G-4 were produced in our laboratory [33]. Isotype-matched mAbs directed against either rat intercellular adhesion molecule or a protein of human cytomegalovirus (pp65) were used as negative control. A homemade rabbit polyclonal antibody against PR3 (Rb anti-PR3) was used as a positive control, and normal rabbit serum was used as a negative control.

Synthesis of peptides

Fifty overlapping peptides were synthesized from the sequence of human PR3 [34]. All overlapping peptides of human PR3 are listed in Figure 1. The overlapping peptides were 15 amino acids long, except for peptides 39 and 49, which contained 16 amino acids, and peptide 38 being 17 amino acids long. The peptides had an overlap of 10 amino acids. At the N-terminus of each peptide, a cysteine residue was present. This residue was either supplementary (c-) or part of the normal PR3 sequence (C). These peptides were synthesized at the peptide center of the research school of infection and immunity, Veterinary Faculty (Utrecht, The Netherlands). Peptides were prepared by automated simultaneous multiple peptide synthesis (SMPS) [35]. The SMPS setup was developed using a standard autosampler (Gilson 221) as described previously [35]. Briefly, standard Fmoc chemistry with in situ PyBop/NMM activation of the amino acid in a fivefold molar excess with respect to 2 mol/peptide PAL-PEG-PS resin (PerSeptive Biosystems, Foster City, CA, USA) was employed. Peptides were obtained as C-terminal amides after cleavage with 90 to 95% trifluoracetic acid (TFA)/scavenger cocktails. The composition and purity of the peptides was checked by reverse-phase high-performance liquid chromatography (RP-HPLC) and ion-trap mass spectrometry.

The sequence of PR3 [34] used to design the peptides in this study differed in three amino acids from the sequence of PR3 [36] used in other studies on binding of

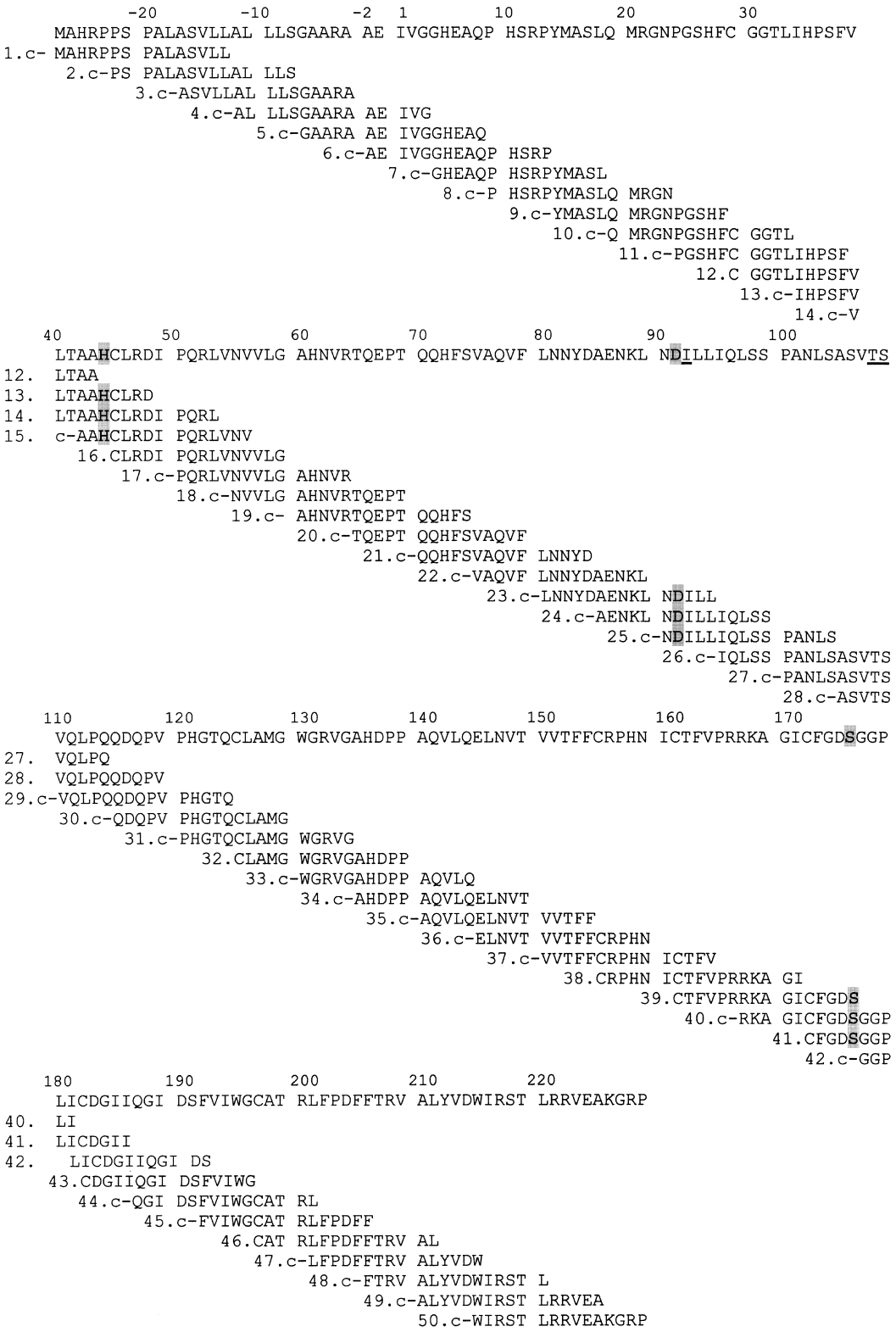

Fig. 1. Diagram illustrating the 50 linear peptides spanning the sequence of proteinase 3 (PR3). Numbering of the amino acids is according to the sequence of PR3 published by Campanelli et al [36]. Residue numbers −27 to −3 constitute the signal sequence; −2 to −1, the prosequence, 1 to 222 the mature form of PR3; residue 222 to 229 the C-terminal domain. Residues of the catalytic triad H^{44}, D^{91}, and S^{176} are marked by gray boxes. Peptide numbers are typed in front of each sequence. At the N terminus of each peptide a cysteine residue is present, this residue was either supplementary (c-) or part of the normal PR3 sequence. The three amino acids that were different in the PR3 sequence used in this study compared with other studies are underlined.

PR3-ANCA to linear peptides of PR3 (abstract; Griffith, *Vasc Diffuse Lung Dis* 13:256, 1996) [21, 22]. This polymorphism in the *PR3* gene has been reported recently [37]. These amino acids are underlined in Figure 1. As two of these variant amino acids, that is, amino acids 108 and 109, were part of an important epitope recognized by PR3-ANCA sera [22]; peptides 26 to 28 were also synthesized with an alanine (A) and threonine (T) at positions 108 and 109.

Peptide ELISA

The cys-peptides were covalently coupled to Covalink NH plates (Nunc, Roskilde, Denmark) to be described elsewhere (van der Zee et al, unpublished data). The NH-reactive groups were modified with 0.5 mmol/L 3-(2-pyridyldithio) propionacid N-hydroxysuccinimide ester (SPDP; Sigma, Zwijndrecht, The Netherlands) into an active thiol group [38]. The active thiol group was used for covalent coupling of peptides with an N-terminal cysteine residue. After 30 minutes, plates were washed in sterile water, and peptides (15 μg/mL), diluted in 0.1 mol/L Tris-HCl, pH 8.0, were incubated for one hour. After washing in sterile water, plates were blocked for 15 minutes in 200 μL/well of 300 mmol/L NaCl, 100 mmol/L Tris-HCl, and 0.25% bovine serum albumin (BSA). Plates were washed in washing buffer (150 mmol/L NaCl, 10 mmol/L Tris-HCl, and 0.05% Tween-20). Next, plates were incubated with either PR3-ANCA sera (1:50), healthy control sera (1:50), Rb anti-PR3 (1:4000), or anti-PR3 mAbs (concentration mAbs 0.1 to 0.5 μg/mL, and PR3G-4 8 μg/mL) in 450 mmol/L NaCl, 100 mmol/L Tris-HCl, 0.25% BSA, 1% normal goat serum, and 0.05% Tween-20. Then either goat anti-human IgG (1:1500; American Qualex, San Clemente, CA, USA), goat anti-rabbit IgG (1:500; Sigma) or affinity-purified goat anti-mouse IgG (1:500; Sigma) was added. All antibodies were alkaline-phosphatase (AP) conjugated. Finally, p-nitrophenyl phosphate (Sigma) in 1 mol/L diethanolamine, pH 9.8, was used as substrate, and color development was measured at 405 nm. One well was always coated with PR3 inactivated by phenylmethylsulfonyl fluoride (PMSF; Sigma) as a positive control, and in one well, peptides were omitted as a blank value. Of the wells coated with PR3 the mean optical density (OD) value + 2 SD of healthy control sera was used as cut-off value for a positive reaction of patient sera. The OD values obtained with this blank were subtracted from the OD values obtained with the peptides. Reaction on peptides was considered positive when the median of the OD values of PR3-ANCA sera or healthy control sera after subtraction of the blanks exceeded an arbitrary value of 0.5. The goat anti-human IgG, goat anti-rabbit IgG, and goat anti-mouse IgG AP-conjugated secondary antibody, when used alone, did not produce any binding with the linear peptides. In one experiment, binding of purified IgG to peptides together with its respective serum was tested. Purified IgG was used at an IgG concentration of 200 μg/mL, which is more or less comparable to the total concentration as present in a 1:50 diluted serum sample as used in the ELISAs.

Statistical analysis

Differences in OD values between PR3-ANCA sera and healthy controls were tested by Mann–Whitney *U* test. Sera of patients with initial presentation or relapse of WG were compared with respect to OD values obtained in the peptide ELISA, units in the direct ELISA, titers of IIF, serum IgG concentrations, levels of C-reactive protein (CRP), and serum creatinine levels by the Mann–Whitney *U* test. Correlation between OD values obtained in the peptide ELISA and units in the direct ELISA, titers of IIF, and serum IgG concentrations were analyzed by the Spearman rank correlation test. The level of significance used was 0.05. All reported *P* values are two sided.

RESULTS

Rabbit anti-PR3 serum

Rabbit serum raised against PR3 bound to three groups of linear peptides of PR3, whereas normal rabbit serum did not bind to any of the linear peptides (Fig. 2A). Rb anti-PR3 bound to the peptides in a highly restricted manner. Peptides 6 and 7, located at the N-terminus of the mature protein, and peptides 28 to 30 and 32 to 34 were recognized. These areas are not located near the active site of PR3. The amino acid stretches recognized were surface accessible [39]. The peptide ELISA with this antibody proved reproducible, as experiments repeated up to three times gave similar results.

Monoclonal antibodies

Anti-PR3 mAbs did not bind to linear PR3 peptides, but did bind to whole PR3. The OD values obtained for the anti-PR3 mAbs on peptides were low and similar to the subclass-specific control mAbs. The goat anti-mouse IgG AP conjugate when used alone gave similar results as anti-PR3 mAbs (data not shown).

Patients and control sera

Twenty-seven patients with WG were studied. All patients had active disease, either during their initial presentation of WG ($N = 16$) or during relapsing disease ($N = 11$). All WG sera recognized PR3 bound to covalink NH plates, whereas sera of healthy controls, except for one, did not (Fig. 3). OD values obtained for PR3-ANCA sera in this assay correlated with the units obtained by direct PR3 ELISA given in Table 1 ($r = 0.52$, $P = 0.006$).

Sera of WG patients and healthy controls recognized linear peptides in a similar pattern (Fig. 4). Interestingly,

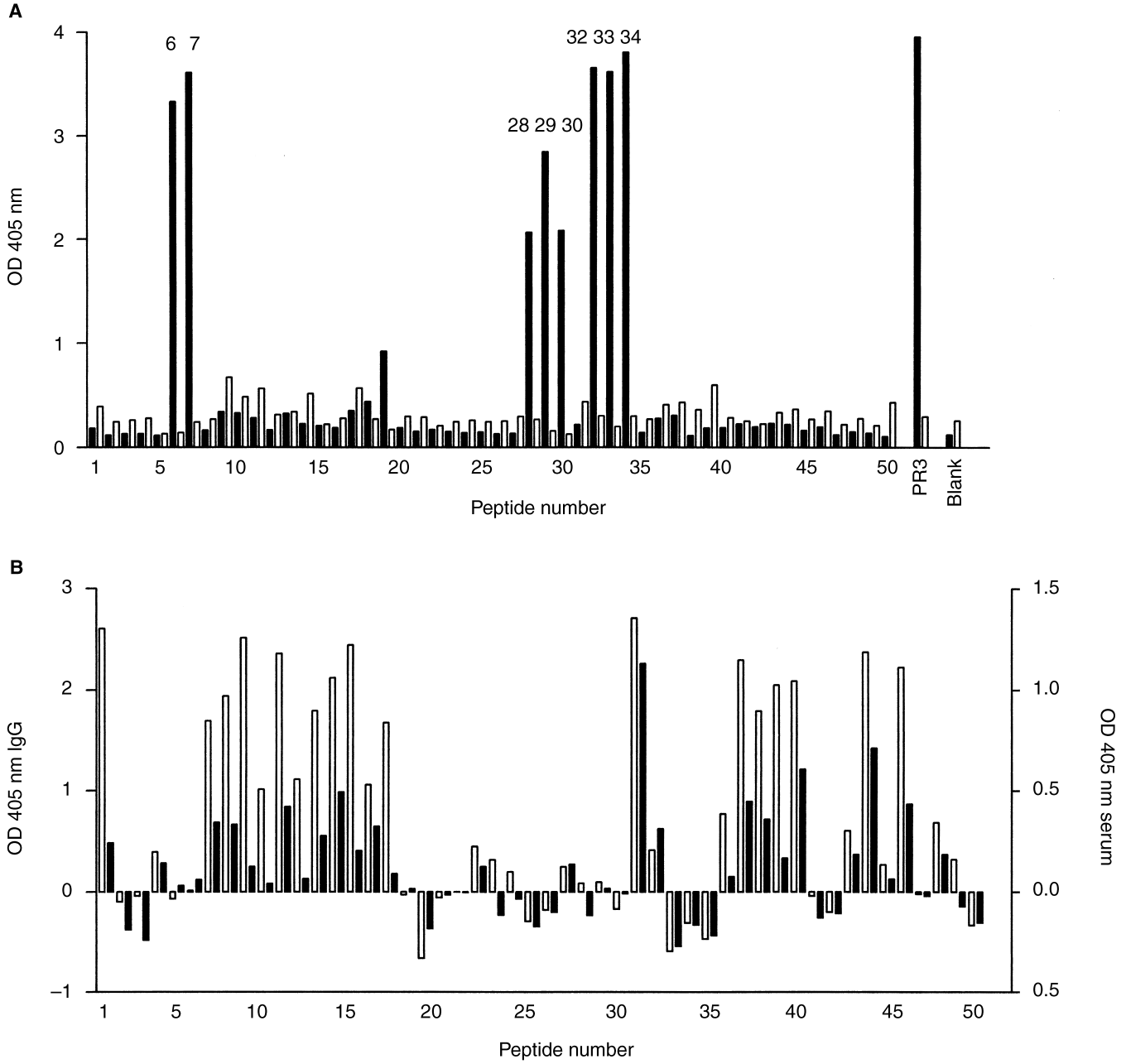

Fig. 2. Peptide ELISA with rabbit serum raised against proteinase 3 (PR3), purified IgG, and serum of a Wegener's granulomatosis (WG) patient. Fifty overlapping peptides (15 μg/mL) spanning the entire sequence of PR3 were coupled on covalink-NH plates. PR3 was coupled as a positive control and wells without peptide (blank) were used as blank values. (*A*) Rabbit anti-PR3 (■) or normal rabbit serum (□) were used at a 4000-fold dilution. (*B*) Serum of WG patient 1 (■) or purified IgG from WG patient 1 (□) was used at a 50-fold serum dilution or 200 μg/mL IgG concentration, respectively. On the *x* axis, peptide numbers are given according to the sequence in Figure 1. On the *y* axis, the OD values measured at 405 nm are depicted.

all sera bound to five distinct peptide areas (peptide numbers 7 to 11, 13 to 14, 31 to 32, 36 to 37, and 39 to 40) and bound to some single peptides (peptide number 1, 28, and 44). This is shown also in Table 2 (second column); reactivity to peptide number 28 was not termed "+" because the median of OD values did not exceed 0.5.

The peptide ELISA proved reproducible, as experiments repeated up to three times with a few patient and control sera gave similar results. The binding of serum sample from patient 1 with its respective purified IgG sample is shown in Figure 2B. Purified IgG bound to the same peptides as its respective serum sample. The similar pattern in binding of WG and control sera could not be explained by binding of the secondary antibody to the

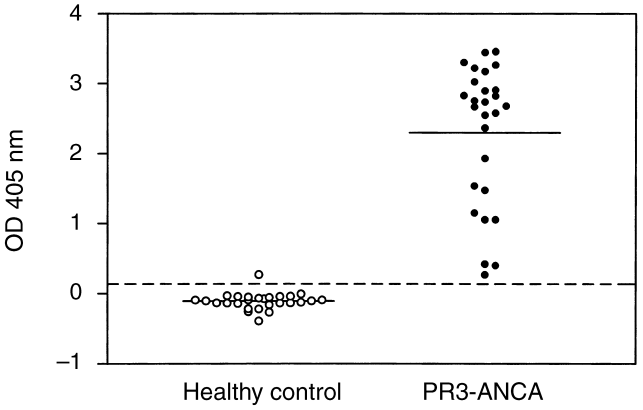

Fig. 3. ELISA on covalink-NH plates with whole PR3 with sera from PR3-ANCA–positive patients and healthy controls. PR3 was coupled on covalink-NH plates as a positive control. PR3-ANCA positive sera (●) or sera from healthy controls (○) were used at a 50-fold dilution. On the *y* axis the OD values measured at 405 nm are depicted. The mean is shown for PR3-ANCA–positive and healthy control sera. Horizontal line represents the mean + 2 SD values of healthy controls, which were taken as cut-off value for a positive reaction.

peptides, as incubation of the secondary antibody alone did not produce binding to any of the peptides. In addition, binding of PR3-ANCA sera to different PR3 peptides did not correlate with levels of total IgG in these sera nor with levels of PR3-ANCA as determined by IIF, direct PR3 ELISA (Table 1), or ELISA with PR3 on NH covalink plates (Fig. 3). Of two patients, the reactions of different serum samples were tested on their binding to linear peptides. The different serum samples preferentially bound to the same peptides for an individual patient (data not shown).

Furthermore, sera of three patients with a relapse of systemic lupus erythematosus and sera of three patients with early rheumatoid arthritis bound to similar peptides as sera from WG patients and healthy controls. The median OD values obtained by these six sera were not enhanced compared with the OD values found in patients with WG and healthy controls (data not shown).

Within the five peptide areas that were bound by WG and control sera, four peptide areas were recognized significantly better by WG sera than by healthy controls (Table 2, third column). This difference in recognition could mainly be attributed to sera of patients with initial presentation of WG (Table 2). Differences between sera of patients with initial presentation or relapse of WG in binding to peptide numbers 7-10, 14-15, 31-32, and 38-40 are depicted in more detail in Figure 5. These differences between sera of patients with initial presentation or relapse of WG were not observed for PR3-ANCA titers determined by either IIF, direct ELISA, or ELISA performed on NH covalink plates with PR3 (Fig. 5). Also, total IgG concentrations or clinical data like CRP levels and/or serum creatinine levels did not differ between both groups. No difference in binding to PR3 peptides was seen between patients with a limited or generalized presentation of WG. The peptide areas recognized by human sera partly overlapped the areas recognized by Rb anti-PR3 (Fig. 2A), as peptides 7 and 32 were bound both by Rb anti-PR3 serum and human sera. Peptides 7 and 32 were not located near the active site of PR3 (Fig. 1).

Two of the peptide areas that were more strongly bound by WG sera than by control sera (peptide numbers 13-14, 39-40) were located near the active center of PR3 at the active site H^{44} and S^{176}, respectively (Fig. 1). Peptide area 7-11 is located at the N-terminus of PR3 (Fig. 1). All peptides recognized were surface accessible [39]. These four identified peptide areas coincided to some extent with earlier described epitopes recognized by PR3-ANCA sera (Table 2; sixth column).

The amino acid sequences of the four peptide areas that were bound more strongly by PR3-ANCA than by control sera were checked on protein similarity in Swissprot using blast 2.0 [40]. The amino acid sequences were highly specific for serine proteases. Serine proteases with the highest amino acid homology with these four identified peptide areas were HLE [41], human medullasin [42], and murine PR3 [43, 44]. In addition, the sequences of the other peptides that were bound by sera also were highly specific for serine proteases.

The sequence of PR3 [34] used to design the peptides in this study differed by two amino acids from the sequence of PR3 [36] used in other studies (abstract; Griffith, *Vasc Diffuse Lung Dis* 13:256, 1996) [21, 22]. These two variant amino acids were part of an important epitope recognized by PR3-ANCA sera [22]. Therefore, binding of sera of WG patients and healthy controls was also tested on peptide numbers 26-28 with T^{108} and S^{109} substituted for alanine and threonine, respectively [37]. Peptides 26-28 variants showed similar binding of PR3-ANCA as peptides 26-28 without amino acid substitutions (data not shown).

DISCUSSION

This study, using overlapping linear peptides spanning the entire sequence of PR3, shows that PR3-ANCA sera of patients with active WG as well as healthy control sera bind to a restricted number of linear PR3 peptides, two of which are located near the active center of PR3. Four peptide areas were bound significantly stronger by sera of WG patients at initial presentation than by healthy controls. The polyclonal rabbit anti-PR3 serum recognized three distinct peptide areas that partly overlapped with the areas recognized by PR3-ANCA sera. Anti-PR3 mAbs did not bind to linear PR3 peptides.

Distinct peptide areas and single peptides were recognized both by sera of WG patients and healthy controls.

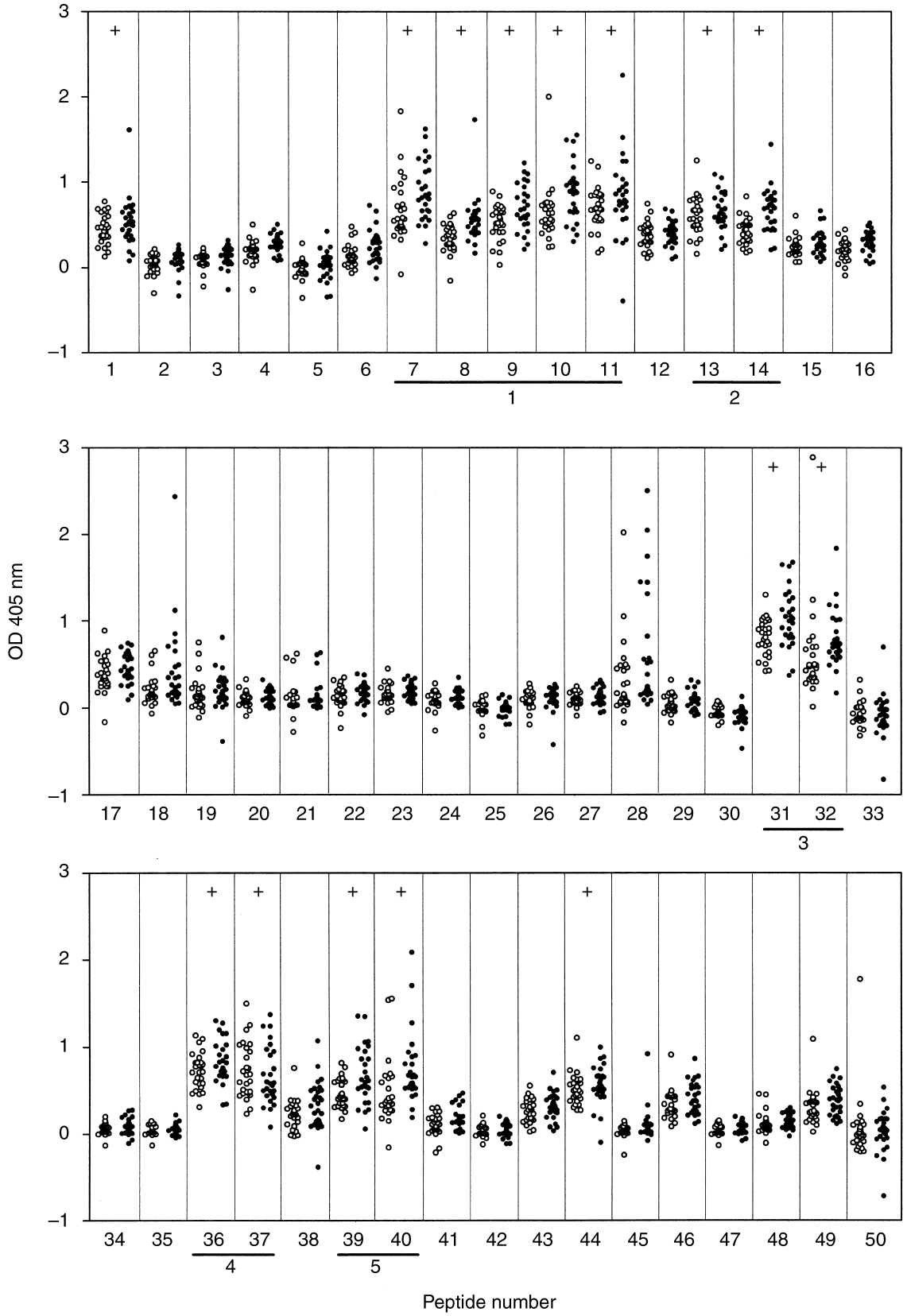

Fig. 4. Peptide ELISA with PR3-ANCA–positive sera of patients with WG and sera of age- and sex-matched healthy controls. Fifty overlapping peptides spanning the entire sequence of PR3 were coupled on covalink-NH plates. Wells without peptide (blank) were used as blank values and subtracted from the optical density (OD) values obtained on wells with peptides. PR3-ANCA–positive sera (●) or sera from healthy controls (○) were used at a 50-fold dilution. On the *x* axis peptide numbers are given according to the sequence in Figure 1. On the *y* axis, the OD values measured at 405 nm are depicted. Reactivity was defined as "+" when median OD values of sera >0.5. Peptide areas recognized by sera are marked with a line and number.

Table 2. Overview on peptides recognized and the significant differences in binding to linear peptides of PR3 between sera of patients with Wegener's granulomatosis and healthy controls

Peptide number	Reaction on peptides				Previous studies
	OD values[a]	All patients[b] $N = 27$	Initial presentation[c] $N = 16$	Relapse[d] $N = 11$	
1	+/−				
2	−	*			
3	−	*	*		
4	−	***	**		
5	−		*		W
6	−	*	*		W/G
7	+/+	**	**		W/G
8	+/−	***	**		W/G
9	+/+	*	**		
10	+/+	**	**		
11	+/+				
12	−				
13	+/+				
14	+/−	***	****		G
15	−		*		G
16	−	**	*		G
17	−				
18	−				
19	−				W
20	−				W
21	−				
22	−				
23	−				
24	−				
25	−				
26	−				
27	−				W
28	−	*			W
29	−				W
30	−	*	*		W
31	+/+	**	**		G
32	+/−	**	*		W/G
33	−				W
34	−				
35	−				
36	+/+				W
37	+/+				W
38	−	*	*		W/G
39	+/−	*	**		W/G
40	+/−	**	*	*	W
41	−				W
42	−				
43	−				W
44	+/−				W
45	−	*			G
46	−				G
47	−				G
48	−	*			
49	−				G
50	−				G

Abbreviations are: W, linear peptides reacting with PR3-ANCA sera as identified by Williams et al [22]; G, linear peptides reacting with PR3-ANCA sera as identified by Griffith et al (abstract; Griffith ME, *Vasc Diffuse Lung Dis* 13:256, 1996)

[a] +/+ = median OD values of PR3-ANCA and healthy control sera >0.5; +/− = median OD values of PR3-ANCA >0.5 and median OD values of healthy control sera <0.5

[b–d] Differences in OD values between PR3-ANCA positive patients and healthy controls (Mann–Whitney *U* test): **P* value between 0.05 and 0.01, ***P* value between 0.01 and 0.001; ****P* value between 0.001 and 0.0001; *****P* value <0.0001

[b] Differences between sera of all WG patients and healthy controls

[c] Differences between sera of patients during their initial presentation of WG and healthy controls

[d] Differences between sera of patients during a relapse of WG and healthy controls

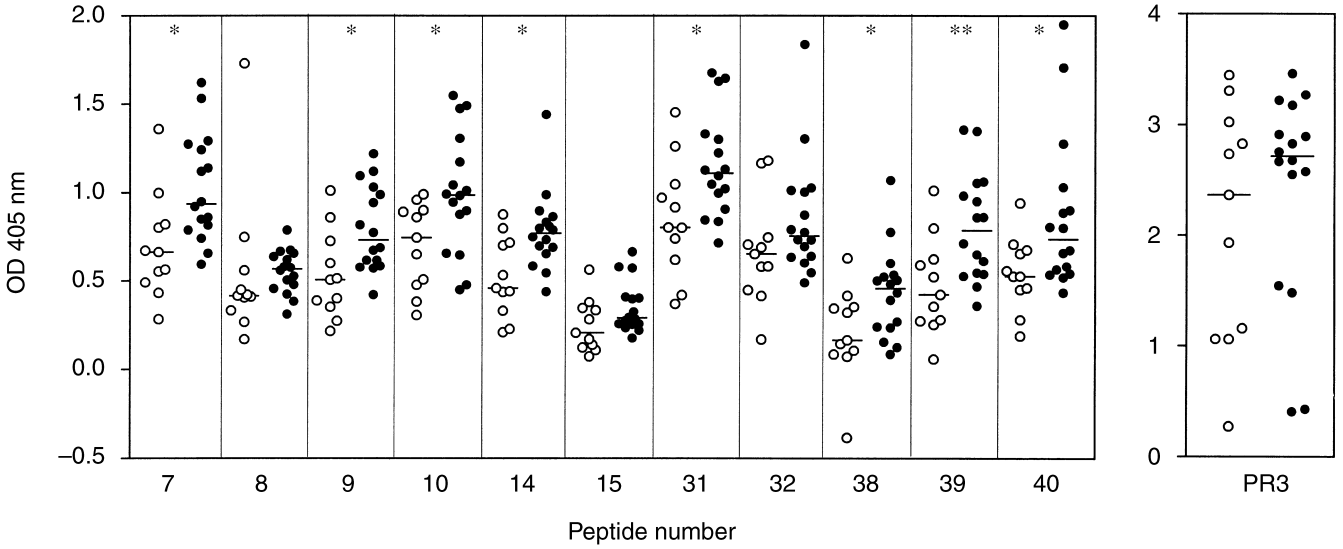

Fig. 5. Peptide ELISA with PR3-ANCA–positive sera of patients at the time of initial presentation or during relapse of WG. Fifty overlapping peptides spanning the entire sequence of PR3 and whole PR3 were coupled on covalink-NH plates. Wells without peptide (blank) were used as blank values and subtracted from the optical density (OD) values obtained on wells with peptides. PR3-ANCA–positive sera from the initial presentation of WG (●) or sera from patients with a relapse of WG (○) were used at a 50-fold dilution. On the *x* axis, PR3 (right segment) and peptide numbers (left segment) are depicted according to sequence in Figure 1. On the *y* axis, the OD values measured at 405 nm are depicted. The median is shown for both groups of sera.

We tested sera at a low, that is, 1:50, dilution, as further dilution of the sera reduced not only background but also specific binding. The low dilution used in this study might suggest aspecific binding of healthy control sera and possibly also of WG sera. We think that this is unlikely since background binding to wells without peptide was lower than binding to peptides, and the OD values presented were derived by subtracting background binding from OD values obtained with peptides. Also, binding occurred to a restricted number of peptides, and no correlation was found between the total IgG levels in sera and the amount of binding to particular peptides. In a previous study, sera of healthy controls diluted 1:800 also bound the same PR3 peptides as WG sera [22], and another study did not find any significant difference between PR3-ANCA and healthy control sera in recognition of PR3 peptides [21]. Thus, specific binding to PR3 peptides occurs by IgG both of WG patients and of controls.

The observation of distinct peptide areas bound by sera from WG patients as well as healthy controls could not be explained by differences in charge characteristics of peptides. Peptides located around the active site are not different from other peptides with respect to charged amino acid components. Peptides located at the C-terminus of PR3 have more positively charged amino acids, but no binding was seen to these peptides.

Four of the peptide areas recognized were bound significantly stronger by WG sera than by sera of healthy controls. These areas on PR3 corresponded to earlier described epitope areas bound by PR3-ANCA (abstract; Griffith, *Vasc Diffuse Lung Dis* 13:256, 1996) [22]. Three of the six epitope regions described by Williams et al [22] and one of the five regions described by Griffith et al (abstract; Griffith et al, *Vasc Diffuse Lung Dis* 13:256, 1996) were not detected in this study. This discrepancy might be due to differences in the PR3 sequence used for peptide construction, the ELISA method used for peptide recognition, peptide length, or the selection of patient and control sera.

Differences in PR3 sequence do not seem relevant, as substitution of the polymorphic amino acids 108 and 109 [37] did not influence recognition by WG or healthy control sera.

With respect to the ELISA system that was used, in our study soluble peptides were synthesized. Purity and sequence of peptides were checked prior to use. Peptides were synthesized with an additional N-terminal cysteine for subsequent coupling to activated ELISA plates, enabling identical coupling of each peptide to the ELISA plate. In previous studies (abstract; Griffith, *Vasc Diffuse Lung Dis* 13:256, 1996) [21, 22], peptides were constructed on pins or membrane and could only be checked for purity and sequence after removal from the pins or membrane. In those studies, this extra check on purity and sequence was probably not performed. Differences in the coupling efficiency of these peptides might influence the recognition by PR3-ANCA sera.

Regarding peptide length, previous studies used peptides of 7 amino acids with an overlap of 6 amino acids [22] or peptides of 10 amino acids with an overlap of 8 (abstract; Griffith, *Vasc Diffuse Lung Dis* 13:256, 1996).

Consequently, in the first study, all possible stretches of 7 amino acids in PR3 and in the second study all possible 9 mers of the PR3 sequence were available as peptides. In the present study, using peptides of 15 amino acids long with an overlap of 10 amino acids, all 11 mers of the PR3 sequence were available. It has been shown that a peptide length of 8 amino acids is sufficient to detect all continuous epitopes [45]. Therefore, none of the previously mentioned studies, as well as our present study, should have missed a linear epitope.

A fourth reason to explain differences in peptide recognition by PR3-ANCA between the present and previous studies relates to the selection of sera. In the current study, sera of WG patients during initial presentation mainly bound the peptide areas. Griffith et al also tested WG patients during initial presentation of disease and PR3-ANCA of all their patients bound to the same restricted peptide regions on PR3 (abstract; Griffith et al, *Vasc Diffuse Lung Dis* 13:256, 1996), as was seen in our study. In another study, sera of WG patients were not characterized based on disease manifestation or duration, and PR3-ANCA of these patients reacted with different peptide areas of PR3 [22]. Thus, the latter discrepancy may be explained, at least in part, by differences in clinical state of WG patients tested.

Apparently, in our study, PR3 peptides are bound by sera of WG patients, as well as by healthy control sera. This was also seen in previous studies [21, 22]. Indeed, natural antibodies reacting with a variety of self-antigens have been detected in serum of healthy individuals [46]. In the present study, the control sera that bound to peptides of PR3 did not recognize the whole PR3 protein, whereas sera of WG patients did (Fig. 3). Evidently, conformation-dependent epitopes on PR3 are preferentially bound by WG sera, whereas linear epitopes on PR3 are equally bound by patient and control sera.

In our study, PR3 peptides were mainly bound by sera of patients at their initial presentation of WG. Studies in systemic lupus erythematosus (SLE) have indicated that the highly diverse autoimmune response in SLE can originate from a single (cryptic) self-peptide without the need for foreign pathogens or molecular mimics [47]. In the present study, it is feasible that upon relapse of WG, apart from affinity maturation, epitope spreading of PR3-ANCA to more conformation-dependent epitopes has occurred, resulting in PR3-ANCA that bind linear peptides with lower affinity. It has indeed been shown that in one patient epitope, spreading of PR3-ANCA occurred during the course of the disease (abstract; Griffith, *Vasc Diffuse Lung Dis* 13:256, 1996).

Two peptides bound by human sera were also bound by a rabbit anti-PR3 serum, indicating that hyperimmunization with PR3 can induce, to some extent, antibodies binding to similar epitopes as sera of WG patients. However, PR3-ANCA sera bound some epitopes overlapping the active site, whereas Rb anti-PR3 did not. Antibodies produced upon immunization are not induced against conserved regions of the antigen, as this is not recognized as foreign. One of the most conserved region of PR3 is around the active site, as the substrate specificity of the proteases is located there. The absence in binding to PR3 peptides of anti-PR3 mAbs might be due to the selection criteria of the hybridomas selecting mAbs that bound to conformation-dependent epitopes.

The peptide areas recognized in our study are located at the N-terminus of PR3, around the active site H^{44} and S^{176}, and in the center of the primary sequence. No epitopes were detected at the C-terminus of PR3 nor in the signal- or prosequence. In the three-dimensional structure of PR3, these peptides come together around the active site and have large homology with murine PR3 [43, 44] and to a lesser extent with HLE [41]. Still, PR3-ANCA do not react with murine PR3 or HLE. Along with the homology in primary sequence, a difference in conformation of the protein could influence the recognition by antibodies. Since PR3-ANCA mainly recognize conformational epitopes [20], this conformational difference might be of crucial importance for the antigen specificity.

Considering that PR3-ANCA mainly recognize conformational epitopes, why do we see a reproducible reaction on linear peptides? Most of the human antibodies recognize conformation dependent epitopes [48]. Conformational epitopes can be identified by x-ray crystallography [49]; nevertheless, some epitopes defined by this method could also be identified by overlapping linear peptides [45]. Indeed, it has been shown that discontinuous linear segments of a conformational epitope can be identified using overlapping linear peptides [50–52].

In summary, our results demonstrate that sera of both WG patients as well as healthy controls bind specifically to a restricted number of PR3 peptides, two of which are located near the active center of PR3. Four of these peptide areas are significantly more strongly recognized by sera of patients with an initial presentation of WG. These data suggest that the autoimmune response to PR3 starts from a universally present response to a restricted number of linear epitopes, and evolves during disease development into responses to conformational epitopes.

ACKNOWLEDGMENTS

This work was supported by the Dutch Kidney Foundation (Research Grant C97.1701). The authors thank Dr. J. Wieslander (Lund, Sweden) for providing the monoclonal antibodies (mAb) 4A3 and 4A5, the late Dr. C.M. Lockwood (Cambridge, UK) for mAb Hz1F12, and Dr. U. Specks (Rochester, MN, USA) for the mAb MC-PR3-2.

Reprint requests to Dr. Ymke M. van der Geld, Department of Clinical Immunology, University Hospital Groningen, Hanzeplein 1, 9713 GZ Groningen, The Netherlands.
E-mail: Y.M.van.der.geld@med.rug.nl

REFERENCES

1. Fauci AS, Haynes BF, Katz P, *et al*: Wegener's granulomatosis: Prospective clinical and therapeutic experience with 85 patients for 21 years. *Ann Intern Med* 98:76–85, 1983
2. Van Der Woude FJ, Rasmussen N, Lobatto S, *et al*: Autoantibodies against neutrophils and monocytes: Tool for diagnosis and marker of disease activity in Wegener's granulomatosis. *Lancet* 1:425–429, 1985
3. Goldschmeding R, Van Der Schoot CE, Ten Bokkel Huinink D, *et al*: Wegener's granulomatosis autoantibodies identify a novel diisopropylfluorophosphate-binding protein in the lysosomes of normal human neutrophils. *J Clin Invest* 84:1577–1587, 1989
4. Niles JL, McCluskey RT, Ahmad MF, *et al*: Wegener's granulomatosis autoantigen is a novel neutrophil serine proteinase. *Blood* 74:1888–1893, 1989
5. Lüdemann J, Utecht B, Gross WL: Anti-neutrophil cytoplasm antibodies in Wegener's granulomatosis recognize an elastinolytic enzyme. *J Exp Med* 171:357–362, 1990
6. Rao NV, Wehner NG, Marshall BC, *et al*: Characterization of proteinase-3 (PR-3), a neutrophil serine proteinase: Structural and functional properties. *J Biol Chem* 266:9540–9548, 1991
7. Cohen Tervaert JW, Van Der Woude FJ, Fauci AS, *et al*: Association between active Wegener's granulomatosis and anticytoplasmic antibodies. *Arch Intern Med* 149:2461–2465, 1989
8. Kallenberg CGM, Brouwer E, Weening JJ, *et al*: Anti-neutrophil cytoplasmic antibodies: Current diagnostic and pathophysiological potential. *Kidney Int* 46:1–15, 1994
9. Muller Kobold AC, Van Der Geld YM, Limburg PC, *et al*: Pathophysiology of ANCA-associated glomerulonephritis. *Nephrol Dial Transplant* 14:1366–1375, 1999
10. Jayne DR, Gaskin G, Pusey CD, *et al*: ANCA and predicting relapse in systemic vasculitis. *Q J Med* 88:127–133, 1995
11. Egner W, Chapel HM: Titration of antibodies against neutrophil cytoplasmic antigens is useful in monitoring disease activity in systemic vasculitides. *Clin Exp Immunol* 82:244–249, 1990
12. Kerr GS, Fleisher TA, Hallahan CW, *et al*: Limited prognostic value of changes in antineutrophil cytoplasmic antibody titer in patients with Wegener's granulomatosis. *Arthritis Rheum* 36:365–371, 1993
13. Hoffman GS, Kerr GS: Rise in ANCA titer: To treat or not to treat. (letter) *Am J Med* 98:102–103, 1995
14. Falk RJ, Terrell RS, Charles LA, *et al*: Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals *in vitro. Proc Natl Acad Sci USA* 87:4115–4119, 1990
15. Charles LA, Caldas ML, Falk RJ, *et al*: Antibodies against granule proteins activate neutrophils *in vitro*. *J Leukoc Biol* 50:539–546, 1991
16. Van De Wiel BA, Dolman KM, Van Der Meer Gerritsen CH, *et al*: Interference of Wegener's granulomatosis autoantibodies with neutrophil proteinase 3 activity. *Clin Exp Immunol* 90:409–414, 1992
17. Daouk GH, Palsson R, Arnaout MA: Inhibition of proteinase 3 by ANCA and its correlation with disease activity in Wegener's granulomatosis. *Kidney Int* 47:1528–1536, 1995
18. Dolman KM, Stegeman CA, Van De Wiel BA, *et al*: Relevance of classic anti-neutrophil cytoplasmic autoantibody (C-ANCA)-mediated inhibition of proteinase 3-alpha 1-antitrypsin complexation to disease activity in Wegener's granulomatosis. *Clin Exp Immunol* 93:405–410, 1993
19. Mulder AH, Stegeman CA, Kallenberg CGM: Activation of granulocytes by anti-neutrophil cytoplasmic antibodies (ANCA) in Wegener's granulomatosis: A predominant role for the IgG3 subclass of ANCA. *Clin Exp Immunol* 101:227–232, 1995
20. Bini P, Gabay JE, Teitel A, *et al*: Antineutrophil cytoplasmic autoantibodies in Wegener's granulomatosis recognize conformational epitope(s) on proteinase 3. *J Immunol* 149:1409–1415, 1992
21. Chang L, Binos S, Savige J: Epitope mapping of anti-proteinase 3 and anti-myeloperoxidase antibodies. *Clin Exp Immunol* 102: 112–119, 1995
22. Williams RC Jr, Staud R, Malone CC, *et al*: Epitopes on proteinase-3 recognized by antibodies from patients with Wegener's granulomatosis. *J Immunol* 152:4722–4737, 1994
23. Leavitt RY, Fauci AS, Bloch DA, *et al*: The American College of Rheumatology 1990 criteria for the classification of Wegener's granulomatosis. *Arthritis Rheum* 33:1101–1107, 1990
24. Cohen Tervaert JW, Huitema MG, Hene RJ, *et al*: Prevention of relapses in Wegener's granulomatosis by treatment based on antineutrophil cytoplasmic antibody titre. *Lancet* 336:709–711, 1990
25. Stegeman CA, Cohen Tervaert JW, De Jong PE, *et al*: Trimethoprim-sulfamethoxazole (co-trimoxazole) for the prevention of relapses of Wegener's granulomatosis: Dutch Co-Trimoxazole Wegener Study Group. *N Engl J Med* 335:16–20, 1996
26. Wiik A: Delineation of a standard procedure for indirect immunofluorescence detection of ANCA. *APMIS Suppl* 6:12–13, 1989
27. van Der Giessen M, Huitema MG, Cohen Tervaert JW, *et al*: A technical note on the routinely performed ANCA detection. *APMIS* 97:37, 1989
28. Cohen Tervaert JW, Goldschmeding R, Elema JD, *et al*: Autoantibodies against myeloid lysosomal enzymes in crescentic glomerulonephritis. *Kidney Int* 37:799–806, 1990
29. Popa ER, Stegeman CA, Bos NA, *et al*: Differential B- and T-cell activation in Wegener's granulomatosis. *J Allergy Clin Immunol* 103:885–894, 1999
30. Leid RW, Ballieux BE, Van Der Heijden I, *et al*: Cleavage and inactivation of human C1 inhibitor by the human leukocyte proteinase, proteinase 3. *Eur J Immunol* 23:2939–2944, 1993
31. Sommarin Y, Rasmussen N, Wieslander J: Characterization of monoclonal antibodies to proteinase-3 and application in the study of epitopes for classical anti-neutrophil cytoplasm antibodies. *Exp Nephrol* 3:249–256, 1995
32. Sun J, Fass DN, Hudson JA, *et al*: Capture-ELISA based on recombinant PR3 is sensitive for PR3-ANCA testing and allows detection of PR3 and PR3-ANCA/PR3 immune complexes. *J Immunol Methods* 211:111–123, 1998
33. Van Der Geld YM, Limburg PC, Kallenberg CGM: Characterization of monoclonal antibodies to proteinase 3 as candidate tools for epitope mapping of human anti-proteinase 3 autoantibodies. *Clin Exp Immunol* 118:487–496, 1999
34. Labbaye C, Musette P, Cayre YE: Wegener autoantigen and myeloblastin are encoded by a single mRNA. *Proc Natl Acad Sci USA* 88:9253–9256, 1991
35. Van Der Zee R, Anderton SM, Buskens CAF, *et al*: Heat shock protein T cell epitopes as immunogenic carriers in subunit vaccines, in *Peptides 1994, Proceedings of the Twenty-Third European Peptide Symposium,* Leiden, ESCOM, 1994, p 841
36. Campanelli D, Melchior M, Fu Y, *et al*: Cloning of cDNA for proteinase 3: A serine protease, antibiotic, and autoantigen from human neutrophils. *J Exp Med* 172:1709–1715, 1990
37. Clave E, Molldrem J, Hensel N, *et al*: Donor-recipient polymorphism of the proteinase 3 gene: A potential target for T-cell alloresponses to myeloid leukemia. *J Immunother* 22:1–6, 1999
38. Carlsson J, Drevin H, Axen R: Protein thiolation and reversible protein-protein conjugation: N-succinimidyl 3-(2-pyridyldithio) propionate, a new heterobifunctional reagent. *Biochem J* 173:723–737, 1978
39. Fujinaga M, Chernaia MM, Halenbeck R, *et al*: The crystal structure of PR3, a neutrophil serine proteinase antigen of Wegener's granulomatosis antibodies. *J Mol Biol* 261:267–278, 1996
40. Gish W, States DJ: Identification of protein coding regions by database similarity search. *Nat Genet* 3:266–272, 1993
41. Sinha S, Watorek W, Karr S, *et al*: Primary structure of human neutrophil elastase. *Proc Natl Acad Sci USA* 84:2228–2232, 1987
42. Aoki Y, Hase T: The primary structure and elastinolytic activity of medullasin (a serine protease of bone marrow). *Biochem Biophys Res Commun* 178:501–506, 1991
43. Jenne DE, Frohlich L, Hummel AM, *et al*: Cloning and functional expression of the murine homologue of proteinase 3: Implications for the design of murine models of vasculitis. *FEBS Lett* 408:187–190, 1997
44. Aveskogh M, Lutzelschwab C, Huang MR, *et al*: Characterization of cDNA clones encoding mouse proteinase 3 (myeloblastine) and cathepsin G. *Immunogenetics* 46:181–191, 1997

45. Geysen HM, Rodda SJ, Mason TJ, *et al*: Strategies for epitope analysis using peptide synthesis. *J Immunol Methods* 102:259–274, 1987
46. Mouthon L, Haury M, Lacroix Desmazes S, *et al*: Analysis of the normal human IgG antibody repertoire: Evidence that IgG autoantibodies of healthy adults recognize a limited and conserved set of protein antigens in homologous tissues. *J Immunol* 154:5769–5778, 1995
47. James JA, Harley JB: B-cell epitope spreading in autoimmunity. *Immunol Rev* 164:185–200, 1998
48. Barlow DJ, Edwards MS, Thornton JM: Continuous and discontinuous protein antigenic determinants. *Nature* 322:747–748, 1986
49. Laver WG, Air GM, Webster RG, *et al*: Epitopes on protein antigens: Misconceptions and realities. *Cell* 61:553–556, 1990
50. Beattie J, Shand JH, Flint DJ: An immobilised peptide array identifies antibodies to a discontinuous epitope in the extracellular domain of the bovine growth hormone receptor. *Eur J Biochem* 239:479–486, 1996
51. Lee MK, Kim KL, Hahm KS: Epitope mapping of preS2 of the hepatitis B virus surface antigen against a conformation-dependent monoclonal antibody using synthetic peptides. *Biochem Mol Biol Int* 40:1077–1085, 1996
52. Levy JB, Coulthart A, Pusey CD: Mapping B cell epitopes in Goodpasture's disease. *J Am Soc Nephrol* 8:1698–1705, 1997